UNIVERSIDADE DE LISBOA
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Aloe species in colorectal cancer therapy: Friend or Foe?

Eduarda Pimenta Veríssimo

Mestrado em Biologia Molecular e Genética

Dissertação orientada por:
Prof. Dr. Ricardo Boavida Ferreira
Prof. Anabela Rosa Bernardes dos Santos Silva

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Abstract

Aloe plants have been suggested to be an important natural source of medical therapy agents (including for cancer) for several years. Throughout the world, pharmaceutical companies and institutions are struggling to isolate its most potent bioactive compounds as a primary means of using its activities. Regardless of the species used, the extraction procedure selected or the lack of specific compounds responsible for any given bioactivity, the fact is that we have been witnessing several debates, disputes and a lot of conflicting results arising on the claimed anticancer properties of *Aloe* species.

During the last decade, several reports demonstrated that a subgroup of matrix metalloproteinases (MMPs) called gelatinases (MMP-2 and especially MMP-9), are largely responsible for colorectal cancer progression/metastasis, suggesting that MMP inhibitors (MMPIs) may be a powerful tool to reduce cancer invasion. Although *Aloe*'s activities suggest they might inhibit MMPs, no studies have related their reported anticancer activity with MMPI activities. Also, although approximately 500 species have been identified so far within the *Aloe* genus, *A. vera* is the most widely studied albeit other species such as *A. arborescens* have also been reported to exhibit similar bioactivities. Hence, the goal of the present study was to compare the anticancer potential of two *Aloe* species, *A. vera* and *A. arborescens* and to ascertain if they are specific MMP-9 and/or MMP-2 inhibitors.

Different types of extraction were tested (100% (v/v) methanol, 50% (v/v) methanol and 100 mM Tris-HCl buffer pH 7) and specific bioactive compounds (proteins, total phenolic compounds, anthraquinones and total carbohydrates) were quantified and compared. Although there were a few variations between species and between extractions, 50% (v/v) methanol was selected as the best extraction procedure. Anticancer activities were measured *in vitro* using the wound healing model assay in the human colon cancer cells HT29, and *A. vera* showed significantly higher inhibitory potencial regarding wound closure. Furthermore, it was also evaluated their effects on gelatinase activities, measured by gelatin zymography and the DQ gelatinase assay. When assessing the total gelatinolytic activity, both species had a similar result of around 20% inhibition. Through the gelatin zimography we demonstrated that both MMP-9 pro-form and active form were inhibited, but only the MMP-2 pro form were inhibited, which corroborates the results obtained with the total gelatinolytic activity assay. The gelatinolytic activity of MMP-9 showed that although both species have the potencial to inhibit specifically this metalloproteinase, A. arborescens have a significantly higher inhibitory effectiveness.

Overall, our data provided clear indication that *A. arborescens* and *A. vera* have indeed potential as inhibitory agents in cancer therapy, being able to reduce colon cancer cell proliferation and invasion, possibly via specific MMP-9 inhibition. *A. arborescens* appears to be a more effective cancer cell invasion inhibitor than *A. vera*. Also, we observed that different types of extraction are important to obtain higher amounts of bioactive compounds and also higher levels of bioactivities. This might be due to a greater amount of extractable bioactive compounds such as anthraquinones. These results open novel perspectives on the mode of action of *Aloe* species in cancer invasion and may provide clues as to why there are so many conflicting results.

Keywords: *Aloe*, cancer, MMP-9, MMP-2, HT29

Resumo

Desde a antiguidade que as espécies do género *Aloe* são consideradas como uma importante fonte natural de compostos terapêuticos. Contudo, nas últimas décadas tem-se verificado um crescimento rápido e exponencial do interesse popular nestas plantas. O *Aloe barbadensis* (mais vulgarmente conhecido como *Aloe vera*) é, entre as espécies deste género, a mais reconhecida e popularizada sendo-lhe atribuídas uma miríade de aplicações e indicações terapêuticas, muitas vezes não fundamentadas cientificamente. Consequentemente, a nível mundial, tanto empresas como instituições farmacêuticas procuram avidamente separar e identificar os compostos bioativos mais potentes entre espécies de *Aloe*, como forma de isolar potenciais bioatividades para aplicar em fármacos ou em produtos vendáveis. Contudo, tem surgido um grande debate entre a comunidade científica sobre o verdadeiro valor terapêutico desta espécie; enquanto alguns trabalhos científicos advogam o seu grande potencial, outros desacreditam totalmente os seus efeitos, alertando mesmo os seus eventuais perigos para a saúde. Existem várias razões possíveis para justificar estas inconsistências nos estudos publicados até à data. *A. vera* é a espécie mais utilizada e conhecida atualmente, sendo o elemento do seu género mais amplamente estudado, apesar de outras espécies tais como *A. arborescens* e *A. ferox* terem sido igualmente descritas como tendo bioatividades semelhantes ou mesmo superiores. Existe também grande inconsistência em relação ao tipo de solventes a utilizar nas extrações, o que pode influenciar a biodisponibilidade e quantidade de compostos ativos. Além disso, cada vez mais parece evidente que a ação terapêutica das plantas de *Aloe* poderá estar associada a uma sinergia entre os vários compostos bioativos presentes nas suas folhas e não apenas à ação de um único composto isolado. Por tudo isto, existe ainda muito trabalho a realizar e muitas questões que deveriam ser abordadas em relação a estas espécies.

Uma das aplicações mais conhecidas e procuradas do género *Aloe* é o seu potencial efeito anti-tumoral. Vários estudos têm comprovado que existe de facto um efeito preventivo e inibidor de vários tipos de tumores induzido pelos extratos e compostos de *Aloe*. Contudo, o mecanismo através do qual estes efeitos são exercidos ainda não está totalmente esclarecido.

Durante a última década, vários estudos revelaram que existe um subgrupo de metaloproteinases de matriz (MMPs), as gelatinases (MMP-2 e MMP-9), que estão envolvidas no processo de metastização. Estes trabalhos têm demonstrado que os seus inibidores (MMPIs) poderão ser uma ferramenta muito útil para reduzir a invasão tumoral. Estas enzimas (sobretudo a MMP-9) também estão fortemente envolvidas em processos de cicatrização e de inflamação. Deste modo, o facto de os efeitos das plantas de *Aloe* estarem associados simultaneamente a atividades cicatrizantes, anti-inflamatórias e anti-tumorais sugere que poderão influenciar o mecanismo de ação da MMP-9 e da MMP-2. Contudo, até à data, ainda não existem estudos que associem o potencial anti-tumoral das espécies de *Aloe* com uma possível inibição das duas gelatinases Assim, neste contexto, no presente trabalho decidiu-se determinar e comparar a atividade anti-tumoral de duas espécies do género *Aloe* bastante conhecidas, *A. vera* e *A. arborescens*, avaliando o seu impacto no crescimento e na migração de células de adenocarcinoma do cólon e determinando se estes efeitos estão ou não associados a alguma influência na atividade das MMP-9 e/ou da MMP-2.

Foram primeiramente avaliadas as diferenças entre as duas espécies de *Aloe*, através da caraterização de diferentes compostos com potencial bioativo já descritas para este género, nomeadamente proteínas, compostos fenólicos, antraquinonas e hidratos de carbono totais. Foi também objetivo testar diferentes métodos de extração e tentar compreender qual o melhor processo de modo a obter o maior teor de compostos bioativos. Todos os compostos foram quantificados em três tipos de extracção: aquosa (em tampão Tris-HCl), hidro-metanólica (50% (v/v) metanol) e metanólica (100% (v/v) metanol).

Os resultados mostraram que, apesar de o teor em compostos bioativos não ter variado de forma marcada entre as duas espécies, *A. vera* apresentou um teor mais elevado em compostos fenólicos totais, enquanto *A. arborescens* apresentou valores superiores em antraquinonas. Os resultados comprovaram também que o método de extração não só influencia fortemente a quantidade de compostos bioativos isolados, como também difere entre as duas espécies. Por exemplo, enquanto *A. vera* mostrou de um modo geral um maior teor em compostos extraídos a 50% (v/v) metanol, no *A. arborescens* estes valores variaram consoante os compostos determinados. Isto é de extrema importância uma vez que muitos estudos utilizam apenas um tipo de extração, independentemente da espécie e do composto a isolar, sendo raros os artigos que utilizam a extração hidro-metanólica.

Em segundo lugar, foi determinado o potencial anti-tumoral dos melhores extratos de *Aloe* em células de adenocarcinoma do cólon humano (células HT29), utilizando os métodos padrão de proliferação celular e de invasão celular. O trabalho efetuado mostrou que as duas espécies estudadas apresentam de facto potencial para serem inibidoras do desenvolvimento tumoral, reduzindo tanto o crescimento celular como a invasão por células HT29. A inibição do crescimento das células tumorais foi dependente da dose e semelhante entre as duas espécies. Estes resultados permitiram ainda selecionar uma concentração EC50, que foi utilizada nos estudos subsequentes. Nos estudos de invasão tumoral, verificou-se que, apesar de as duas espécies terem tido atividades similares na proliferação celular, na invasão *A. arborescens* revelou ser a espécie com maior atividade, atingindo valores de inibição semelhantes ao conhecido inibidor de MMP-9, doxiciclina. Estes resultados sugerem que as pequenas diferenças de compostos bioativos observadas anteriormente, nomeadamente o maior teor em antraquinonas, poderão estar associados a um maior potencial de inibição por parte de *A. arborescens*.

Uma vez que, segundo a literatura, uma maior inibição na invasão está normalmente associada a uma redução na atividade das gelatinases MMP-9 e MMP-2, foi subsequentemente avaliada a atividade destas duas enzimas no meio extracelular das células HT29, após exposição aos extratos de cada uma das espécies de *Aloe.* Para tal, foi utilizado o método da DQ gelatina que avalia a atividade gelatinolítica total e a zimografia de substrato, que separa as MMP-9 das MMP-2 num gel SDS-PAGE, permitindo a avaliação da atividade específica de cada uma. Os resultados das atividades das gelatinases corroboraram que os extratos das duas espécies possuem de facto capacidade de inibição das gelatinases, mas com maior especificidade para a MMP-9 do que para a MMP-2. De forma a testar se esta inibição ocorre por influência direta na enzima, foi ainda efetuado um ensaio de inibição enzimática com uma MMP-9 comercial na presença da DQ gelatina. Ambas as espécies mostraram inibir a gelatinase MMP-9. Contudo, o extrato de *A. arborescens* mostrou maior atividade inibitória que o de *A. vera*. Não obstante, a inibição da MMP-9 não foi tão elevada como a verificada nas zimografias do meio extracelular das células HT29. Deste modo, os resultados sugerem que poderão existir outros mecanismos de ação que não apenas a inibição direta da enzima.

Em conclusão, o trabalho efetuado mostrou que as duas espécies estudadas de *Aloe* apresentam de facto um potencial para serem inibidoras do desenvolvimento tumoral, inibindo tanto o crescimento celular como a invasão. Os resultados mostram também que esta inibição pode estar relacionada com uma redução específica da atividade da MMP-9 e da MMP-2.

Apesar de terem concentrações semelhantes das várias classes de compostos bioativos, *A. arborescens* parece ser um inibidor da invasão tumoral mais eficaz do que *A. vera*. Isto pode ser devido a uma maior quantidade de compostos bioativos individuais, como as antraquinonas presentes em *A. arborescens*, ou à presença de compostos específicos ainda não identificados nessa espécie. Os resultados aqui apresentados mostram igualmente que a utilização de espécies de *Aloe*, quer seja na prevenção ou na terapia tumoral, deve ter sempre em consideração o tipo de extração, devendo esta adaptar-se de acordo com a espécie e o objetivo em mente; de outro modo podem não ser obtidos os resultados mais realistas e reveladores do verdadeiro potencial da espécie.

De um modo geral, *A arborescens* parece ser uma alternativa mais eficaz do que *A. vera* no caso da inibição e da invasão celulares, sugerindo um elevado potencial de utilização como coadjuvante em terapias tumorais, ou ainda na prevenção da formação de metástases em tumores já estabelecidos. Uma vez que a inibição direta das enzimas parece não ser o seu único mecanismo de ação, mais estudos deveriam ser efetuados para determinar a sua toxicidade e efeitos. Não obstante, os resultados obtidos neste trabalho abrem as portas para novos trabalhos sobre a inibição de MMPs por parte das espécies de *Aloe*, chamando a atenção para a necessidade de uma reavaliação do tipo de metodologias de extração utilizados, bem como as espécies utilizadas e respetivos alvos terapêuticos.

Palavras-chave: *Aloe*, cancro, MMP-9, MMP-2, HT29

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List of Abbreviations

BSA – Bovine serum albumin

CRC – Colorectal cancer

DNA – Deoxyribonucleic acid

DQ – Dye-quenched

EC50 – Half maximal effective concentration

ECM - Extracellular matrix

EDTA - Ethylenediamine tetraacetic acid

FBS – Fetal bovine serum

ISA – Instituto Superior de Agronomia, Universidade de Lisboa

MIC – Minimal inhibitory concentration

MMP – Matrix metalloproteinase

MMPI – Matrix metalloproteinase inhibitor

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PVPP - Polyvinylpolypyrrolidone

PBS – Phosphate buffered saline

RPMI – *Roswel Park Memorial Institute* Medium

SD – Standard deviation

SDS – Sodium dodecyl sulphate

SDS-PAGE – Sodium dodecyl sulphate-polyacrilamide gel electroforesis

TCA – Trichloroacetic acid

TIMP – Tissue inhibitors of metalloproteinase

TRIS – 2-Amino-2-hydroxymethyl-propane-1,3-diol

1 - Introduction

1.1 The *Aloe* genus: century-old known medicinal plants

The genus *Aloe* has been one of the best known for medicinal purposes in several cultures for millennia and its uses are referred to as early as in Egyptian papyri or the *Materia Medica* of Dioscorides (Fig.1.1) and the Colloquies of Garcia de Orta [1, 2]. Nonetheless, it was not until the last decades that public interest in *Aloe* species grew tremendously, with such a vast array of proclaimed properties and applications, ranging from burns, skin damage, edema, pain and gastric ulcers, to cancer that it has been labeled a "cure-all miracle" plant. This boosted the investigator's interest on this plant and consequently, a considerable amount of research about the various components of *Aloe* began to arise, aiming to find out more about their properties and to pinpoint specific compounds which may be used/commercialized as pharmacological agents [3]. Indeed, *Aloe* extracts have been reported to possess anti-inflammatory, anti-diabetes, cellular protection, restoration, and mucus-stimulating activities, among others, which might be related to its broad-range effects [4- 7].

Figure 1.1 - Ancient Greek Dioscorides recorded his use of *Aloe* to treat war wounds as well as piles *(Image: Vienna Cod.med.gr.1, fol.15r/ONB)*

Today, *Aloe barbadensis* (herein referred to as *Aloe vera*) is unquestionably the most popular species of *Aloe* used worldwide, often referred to as the one with the highest bioactivities in anecdotal reports. However, there are approximately 500 *Aloe* species documented, from which only a marginal fraction has recorded ethnomedicinal uses [8]. As a result of its huge medicinal demands and the morphological similarities among several species, there is a high probability that certain species may often be erroneously identified for different medicinal uses. So in addition to the increasing interest in the phytochemical and pharmacological potential of *Aloe*, the regular evaluation and re-evaluation of existing bodies of knowledge are deemed necessary to provide an up-to-date information while identifying the current research gaps. In Portugal, access to different *Aloe* species is difficult, as they are mostly found in the African continent [8], but Instituto Superior de Agronomia (ISA) has the largest collection of *Aloe* species in Europe, counting with more than 80 species, collecting species from Africa, identifying new taxa and establishing and maintaining cultures in the ISA´s botanical gardens.

1.1.1 Anatomy

The genus *Aloe* belongs to the *Xanthorrhoeaceae* family and subfamily *Asphodeloideae*, characterized by plants presenting a rosette of large and succulent leaves. The *Aloe* species are relatively similar regarding their anatomy. All plants have boat-shaped fleshy leaves with spiny margins, arranged in rosettes or spirals [9, 10]. These plants usually produce racemose inflorescences with tubular flowers, with a yellow orange, pink or red color. They flower mainly during the winter season and produce large quantities of small, air-borne seeds, although there are many species that can also propagate asexually [9, 10]. Although *Aloe* plants are relatively similar in structure, they exhibit a wide morphological variability and various growth forms [11], ranging in size from dwarf species, of only a few cm high to giant, 20 m tree *Aloe*s [9, 12].

The leaf structure of the *Aloe* plant can be separate into three different components, from the exterior portion to the most interior: 1) a green rind or cuticle, with multiple layers interspersed with chloroplasts, 2) a region composed by vascular bundles with three tubular structures, which secretes a

latex or sap when cut, and 3) an inner pulp or gel [13]. The gel or pulp comprises the major part of the leaf's volume and is composed by large thin-walled parenchyma cells responsible for the clear mucilaginous aqueous extract obtained from this part of the plant [14, 15] (Fig.1.2). Carbohydrates synthesized in excess are transported via phloem to these cells which also store water, minerals and malic acid. Thus, the parenchymal cells serve as a water and energy reservoir for the plant. The latex (yellow substance visible in Figure 1.2) is restricted to the margins of the leaves, suggesting that it may be a source of secondary metabolites [15]. Although there are several articles highlighting the pytochemical composition of *Aloe vera*, little is known about other species, such as *A. arborescens* and *A. ferox*, which have also been the target of several assays in the last years [16-19].

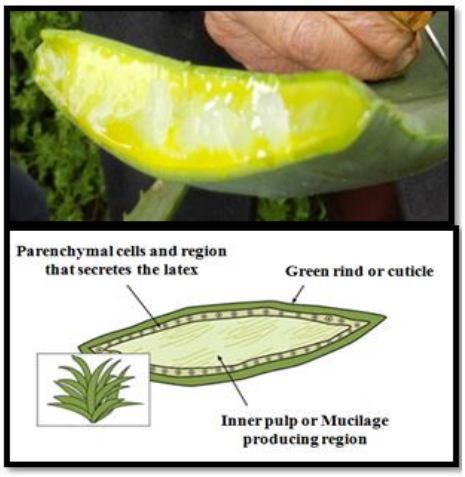

Figure 1.2 - Components of the typical leaf structure of an *Aloe* plant. (Adapted from http://www.niehs.nih.gov/health/topics/agents/aloe/index.cfm)

1.1.2 Reported groups of bioactive components and properties in the *Aloe* genus

According to Surjushe *et al*. (2008) [20] *Aloe vera* is by far the most thoroughly characterized species within *Aloe* genus, contains many potentially active constituents: vitamins, enzymes, minerals, sugars, lignin, saponins, salicylic acids and amino acids [5, 22, 27]. Many of these compounds are responsible for some pharmacological activities described, such as aloin, *Aloe*sin, *Aloe*nin, *Aloe*resin and *Aloe*-emodin, among others [8, 23]. It is known that these compounds differ greatly among species, and some of them can be toxic and extremely allergenic [23, 24].

Below is a list of the main compounds present in *A. vera*:

1. ***Vitamins*:** vitamins A (β-carotene), C and E, which are antioxidants, are present in *A. vera*, which also contains vitamin B12, folic acid, and choline. Antioxidant compounds aid organisms coping with oxidative stress [7, 20, 21].
2. ***Enzymes***: *A. vera* contains several enzymes such as alkaline phosphatase, amylase, bradykinase, carboxypeptidase, catalase, cellulase, lipase, and peroxidase. Bradykinase helps to reduce excessive inflammation when applied to the skin topically, while others help in the breakdown of sugars and fats [7, 20].
3. ***Minerals:*** *A. vera* is a good dietary source of calcium, chromium, copper, selenium, magnesium, manganese, potassium, sodium and zinc. These elements are essential for the proper functioning of various enzyme systems in different metabolic pathways and a few also participate in the organism's antioxidants defenses [7, 20].
4. ***Sugars:*** *A. vera* provides essentially monosaccharides (glucose and fructose) and polysaccharides: (glucomannans/polymannose). These are derived from the mucilage layer of the plant and are known as mucopolysaccharides. The most prominent monosaccharide is mannose-6-phosphate, and the most common polysaccharides are glucomannans [β-(1,4)-acetylated mannan]. Acemannan, a prominent glucomannan has also been found. Recently, a glycoprotein with antiallergic properties, called alprogen and a novel anti-inflammatory compound, C-glucosyl chromone, have been isolated from *Aloe vera* gel [23, 25, 26, 29].
5. ***Anthraquinones:*** *A. vera* provides many anthraquinones, which are phenolic compounds traditionally known as laxatives [8, 20]. Aloe-emodin, *Aloe*sinand barbaloin are associated to

decelerate tumor growth and had been reported to have chemo-preventive effect in cancer cells [21].

6. ***Fatty acids:*** *A. vera* provides 4 plant steroids, cholesterol, campesterol, β-sisosterol and lupeol. These exhibit anti-inflammatory action and lupeol also possesses antiseptic and analgesic properties [20].
7. ***Hormones:*** *A. vera* auxins and gibberellins help in wound healing and have anti-inflammatory action [7, 20].
8. ***Others:*** *A. vera* contains salicylic acid that possesses anti-inflammatory and antibacterial properties. Lignin, an apparently inert phenolic polymer, when included in topical preparations, enhances penetrative effect of the other ingredients into the skin. Saponins are the isoprenoid soapy substances which form about 3% of the gel and have cleansing and antiseptic properties [20].

1.2 *Aloe* as a pharmacological tool for cancer treatment

Albeit the widespread use of popular traditional medicine throughout the globe, *Aloe* species are used for several ailments, ranging from immunomodulatory, anti-inflammatory, antiulcer, antimicrobial and antifungal activity [7, 14, 18, 30]. One of its most popular attributes is proclaimed anticancer activities, which have also been one of the most studied activities in this genus. Reported studies have shown the plant´s effectiveness towards various cancer types such as liver, colon, duodenal, skin, pancreatic, intestinal, lung and kidney, and these studies have been confirmed by several experimental *in vitro* and *in vivo* studies [31, 32]. *Aloe vera* extracted components have been proven to decelerate tumor growth, especially the anthraquinones like *Aloe*sin, *Aloe*-emodin and barbaloin [33]. These components have been reported to exert a chemo-preventive effect through the regulation of several enzymes in the cell [33]. *Aloe*-emodin can induce cell apoptosis through S-phase arrest in a dose- and time-dependent manner [33-36]. Acemannan extracted from *A. vera* and *A. arborescens* parenchyma cells have an immunomodulatory activity and stimulate necrosis and regression of tumors [25, 26, 37]. This polysaccharide also shows to have a significant effect on wound healing, by accelerating the process and increasing epithelialization in burns [37].

1.3 Matrix metalloproteinases-2 and -9 as targets for cancer therapy

Matrix metalloproteinases (MMPs) are a multigene family of zinc-dependent extracellular matrix remodeling endopeptidases involved in several pathological processes like carcinogenesis, inflammation and auto-immune disorders. Their regulatory activity was found to be fundamental for tumor growth and for the multistep processes leading to invasion and metastasis [38, 39]. Several studies demonstrated that a specific group of MMPs called gelatinases (MMP-2 and MMP-9) are highly related to cancer invasion both by degradation of the cellular matrix [40-43], and the subsequent release of cancer cells through proteolysis [44]. These gelatinases contain a unique set of three repeats of fibronectin that facilitate degradation of gelatinous substrates such as elastin, collagen type I and IV, gelatin and fibrinogen (Fig.1.3) [45-47].

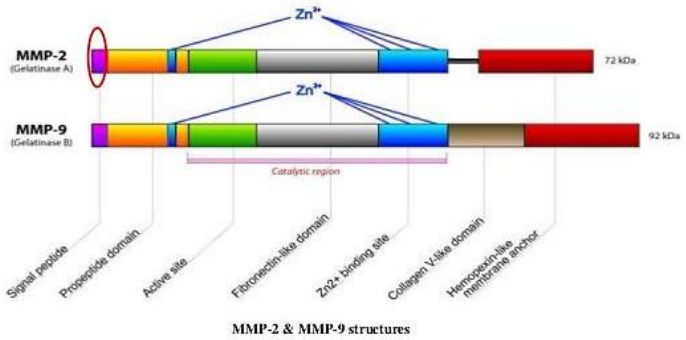

Figure 1.3 - MMP-2 and MMP-9 structures (Adapted from 3http://arthritis-research.com/content/figures/ar2532-1-l.jpg).

Regarding the wide variety of molecules that can be influenced by MMPs activity, their proteolytic function can result in both local and long-distance effects regarding the original site of product cleavage [48]. Also, considering the range of proteins influenced by MMPs, their pivotal role in a cancer scenario can be easily comprehended.

1.3.1 MMP inhibition aimed at cancer prevention/treatment

Since death of cancer patients is usually caused by metastization rather than caused directly by the primary tumor itself [49, 50], and considering that cancer cell invasion is a key element in metastasis and requires MMPs for focalized proteolysis [44, 51-53], in the last decades most anticancer drugs were designed to target MMP activity as MMP inhibitors (MMPIs), which became an important branch of research in both academic and industrial settings [54, 55]. So far, numerous MMP inhibitors have been tested in *in vitro* assays and in animal models, as well as in clinical trials [56-58]. However MMPIs with high specificity and low side effects have been very hard to find, and most clinical trials yielded unsatisfactory results [50, 56, 57, 59]. A major problem associated to unspecific inhibition of MMP-9 is that this enzyme is involved not only in various diseases but also beneficial in remodeling and scar tissue, so its complete inhibition causes several complications in the human body [60, 61]. A distinct and more recent strategy in the search for novel MMP-9 inhibitors is to 'look' among the multitude of natural products that nature placed at our disposal. Under this context, there has been an intense search on various biological sources to develop novel anti-cancer drugs, particularly plant-food products or bioactive plant compounds [62, 63].

1.3.2 Can *Aloe* compounds target gelatinases MMP-9 and MMP-2?

Since MMP-9 and MMP-2 have been recently closely associated to inflammation, wound closure and also the formation of metastasis in several types of cancer [64-68], the wide range activities of *Aloe* suggest its target might be a link between these two diseases, i.e. inflammation and cancer. But although it is known that MMPs play a critical role in both [69-71], to our knowledge there has been no link established between MMP-9 inhibition induced by *Aloe* and cancer. So far, *Aloe*'s anticancer activity and antineoplastic properties is related to three main mechanisms: antiproliferation, immunostimulation and antioxidant effects. The antiproliferative action is known to be determined by anthracenic and antraquinonic molecules, while the immunostimulating activity is mainly due to acemannan [16].

Nonetheless, regarding inflammation, *Aloe vera* extracts are known to down regulate metalloproteinases expression, specifically MMP-9, which actively participates in the degradation of the extracellular matrix (ECM) when recruited by cytokines to inflammation sites [72]. So it seems

plausible that *Aloe* species might inhibit MMP-9 in cancer scenarios. This type of studies can be useful to ascertain the real effect of *Aloe* species on cancer and also lead to the identification of novel anticancer strategies which can be used in prevention, for assisting chemotherapy, or even to prevent re-incidence after surgery. Furthermore, the analysis of MMP inhibition may also be a novel perspective which can help to understand the mechanisms of action observed in *A. vera*.

1.4 The *Aloe vera* paradigm: friend or foe?

There is an intense debate whether the so called miracle plant exhibits all the proclaimed benefits. For example, whilst many papers described its anticancer properties [28, 31, 32, 73], other authors suprisingly claim *A. vera* is in fact carcinogenic over a specific dose [23] or alert that some specific compounds in *Aloe* may be severely toxic if not taken under caution [26]. Some countries such as the USA have even forbidden the use of *Aloe* for consumption because of the lack of consensus towards this species' activities (although it is still allowed as a topic agent). This dramatic imposition is highly disputed by communities worldwide, as the empirical experience and anecdotal results suggest that *Aloe* has a high efficiency. This results in a generalized unsupervised use of *Aloe*, without a scientific background. Furthermore, the phenotype similarities among species are extremely high, which induces people to use often the wrong species. Therefore, the current unsupervised use of *A. vera* can result in cases of allergies and toxic effects, and in scientific studies, can originate an erroneous evaluation of the real effective benefits of the species [71, 74, 75].

Many reasons can be advanced to explain these contradictory reports on *A. vera*: first and foremost, the overall misidentification of the *Aloe* genus' species, not only because they can be erroneously mistaken with *A. vera,* but also because different species with potentially different and stronger bioactivities might be being neglected. In fact, ancient medical texts refer to *A. perryi* or *A. ferox* instead of *A. vera* [1, 64] for medicinal effects. Current research also supports other *Aloe* species do contain more potent bioactivities than *A.vera* [8]. Nonethless these species continue to be neglected. A search on PubMed (29.09.2016) gave a total of 2689 hits for *Aloe* (more than 96% involving *A. vera*) with only very few studies focusing on species other than *A. vera* (e.g. *A. arborescens* and *A. ferox*). So it seems plausible to infer that we have barely scratched the surface in exploring these other species as sources of pharmacological agents [8].

Secondly, in the last decades an effort has been made to isolate phytochemicals that can be used as a single chemical entity and as an alternative to synthetic drugs. However, in the last few years a different perspective has emerged suggesting that using crude and/or standardized extracts as opposed to single compounds might be an advantage since each component has its major effect when acting synergistically with other components in the plant [30, 75, 76]. In this context, it also important to refer that there is an overall lack of consistency concerning the type of extractions to use. Some reports refer to aqueous extracts [77] whilst others use alcoholic or organic solvents such as methanol or acetone [78, 79]. Since the main phytochemical groups range from phenolic compounds to carbohydrates, proteins, organic and inorganic compounds as well as vitamins [7, 21, 82-84], it becomes important to test different types of extractions to identify which provide the highest amount of bioactive compounds and allows the better synergy among them.

1.5 Objectives

The genus *Aloe* has a high therapeutic potential and is often described as a cancer inhibitor. However, the mechanisms by which different species exert their effects it is not clear and there are many conflicting results. Also, less than 20 *Aloe* species have been evaluated under this perspective and their efficacy is highly debated throughout the scientific community. The fact that their effects are

associated with wound healing, anti-inflammatory and antitumor activities suggests that they may influence the activity of the gelatinases MMP-9 and MMP-2. However, there is a generalized lack of consensus towards the type of extraction one should use, the mechanism of action responsible for the proclaimed benefits of *Aloe* and furthermore, if *A .vera* is indeed the best species for cancer prevention / treatment. Hence, in this work, we aimed to evaluate and compare the antitumor activity of two well-known species of the genus *Aloe*: *Aloe vera* and *Aloe arborescens* and to ascertain if it is related to MMP inhibition. With that in mind, we set out to evaluate their content in several classes of potentially bioactive compounds, using different extraction procedures and to assess their impact on the growth and migration of colon adenocarcinoma cells, as well as their effect on MMP-9 and MMP-2 activities.

The specific goals were:

1 – To compare the potentially bioactive compounds in both species, *Aloe vera* and *Aloe arborescens*;

2 – To determine which is the best extraction procedure to obtain higher amounts of the bioactive compounds;

2 – To test the effect of the same extracts in colon cancer cells, aiming to evaluate specifically cell proliferation, cell adhesion and cell invasion rates.;

4 – To test the potential of *Aloe* extracts as inhibitors of the gelatinases MMP-9 and MMP-2, using enzymatic and zymographic assays;

3 – To identify possible mechanisms of action in both *Aloe*s, selecting the better extraction procedures and the best species to be used in prevention and therapy in colon cancer.

2 – Materials and Methods

2.1 Species selected and collection of plant samples

Leaves of *A. vera*, and *A. arborescens* were collected from the existing Collection of *Aloe* Plants of the Instituto Superior de Agronomia (Universidade de Lisboa), probably the biggest collection of this genus outside Africa, with over 80 species. All selected individuals had several years of existence and were not in the flowering season. Leaf samples were collected from at least three separate individuals.

2.2 Preparation of the leaf extracts

Fresh leaves were washed with running tap water and soap and chopped into small fragments of approximately 20 g each. Three different extraction methods were performed using different solvents: 1) 100% (v/v) methanol, 2) 50% (v/v) methanol and 3) 100 mM Tris-HCl buffer, pH 7. All extractions were performed grinding a fragment of 20 g with the respective solvent using an ULTRA-TURRAX T25 (IKA®Labortechnik) grinder, followed by agitation for 4h at a 4º C. Both extracts containing methanol were evaporated in a bath at 60˚C (Kottermann) whereas the aqueous extracts were desalted through filtration using 3 kDa membrane centricons and centrifuged at 2.000 *g*. All extracts were then lyophilized (Edwards Modulyo EF4) for 24h and the obtained powder was weighted and stored at -20ºC.

2.3 Quantification of bioactive compounds

2.3.1 Proteins

Protein quantification was performed using the standard Bradford method as described by Bradford *et al.* (1976) [85]. The samples were read in a spectrophotometer Syenery HT, Bio-TEK at 595 nm and bovine serum albumin was used as standard. All samples used in protein quantification were treated with 1:4 (w/w) polyvinylpolypyrrolidone (PVPP) to eliminate the phenolic compounds that could interfere with the extraction of soluble proteins [86].

2.3.2 Phenolic compounds

The phenolic compounds were quantified using the Folin-Ciocalteau reagent using gallic acid as standard. The lyophilized powder (corresponding to 20g of fresh leaves) was treated with 10 µL of 70% (v/v) acetone, 10 µL of 0.5% (v/v) acetic acid and 80 µL of 7% (w/v) sodium carbonate. Subsequently, a volume of 100 µL of Folin-Ciocalteau was added to the solution and the mixture was vortexed. The solution of 200 µL was incubated for 8 min at room temperature and the absorbance was read in a Syenery HT Bio-TEK spectrophotometer at 765 nm [87].

2.3.2.1 Anthraquinone

To 300 µL of the previous extracts, 300 µL of pure benzene were added and the resulting solution incubated with agitation at 0˚C bath for 30 min. It was then centrifuged at 4.500 g for 20 min at 4˚C in a Beckman J2-21M/E centrifuge. To the recovered supernatant 500 µL of 10% (v/v) ammonia solution were added and the absorbance read at 515 nm using *Aloe blue curacao* aloin as a standard [88].

2.3.3 Total carbohydrates

Sugar quantification was performed utilizing the phenol/sulfuric acid method and mannose was used as the standard. To the previous extracts a 4% (v/v) phenol solution was added in a proportion of 1:5 and then incubated 5 min at room temperature. Afterwards, a 1:40 proportion of sulfuric acid was added and the absorbance read at 492 nm in the equipment mentioned above [89].

2.4 *In vitro* colon cancer cell assays

2.4.1 HT29 cell culture

The human colon adenocarcinoma cell line, HT29 (ECACC 85061109), obtained from a 44 year old Caucasian female, was used throughout this work. HT29 cell lines were maintained in RPMI medium supplemented with 10% (w/v) of heat-inactivated fetal bovine serum (FBS) and 200 mM glutamine, $2x10^4$ UI/mL penicillin and 20 mg/mL streptomycin at 37 °C, in a humidified atmosphere of 5% (v/v) CO_2.

2.4.2 Cell proliferation assay

HT29 cultured cells were seeded on 96-well plates (2 x 10^4 cells/well) and *Aloe* samples were added to the growth media in different concentrations, and incubated for 24 h. After each treatment, the extracellular media was collected, and the wells were washed with PBS to remove unattached cells. Cell proliferation and viability was determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Carmichael *et al*. (1987) [90].

2.4.3 Minimal inhibitory concentrations (MICs)

Minimal inhibitory concentrations (MICs) were assessed in sterile 96-well plates (Greiner Bio-one, Germany), using the micro dilution method as described before [91]. Briefly, 50 µL of RPMI medium was added to each well. Then, 50 µL of each *Aloe* sample (at a 100 µg/mL protein) was added to the first well and serially diluted 1:2 to each adjacent well, up to 10 dilutions. Subsequently, 50 µL of the HT-29 cell suspension with a concentration of $2x10^5$ cells/mL was added to the wells. Two controls were performed: 1) 50 µL RPMI medium + 50 µL cell suspension, 2) 100 µL RPMI medium. Plates were incubated for 24 h, at 37 ºC, and cell growth was measured by the MTT assay [90].

2.4.4 Cell migration assay

For cell migration analysis, the wound healing assay was performed. HT29 cells ($5x10^5$ cells/well) were seeded in 6-well plates and allowed to reach to 80% confluence. Wounds were performed by making a scratch across the cell monolayer to create an open gap, mimicking a wound. Cells were then washed twice with PBS to remove floating debris. Each well was subsequently filled with fresh media containing the samples under study, in a concentration of 100 µg/mL and allowed to grow for 48 h. The invaded area after 48 h was calculated in each treatment and compared to the initial area at 0 h, to determine the area covered by migrating cells into the denuded zone at the beginning of treatment. This comparison allowed us to assess the inhibitory effect (if any) exerted by each protein fraction on the HT29 cell migrating capacity.

2.5 MMP-9 and MMP-2 catalytic activities

2.5.1 Gelatinolytic activity

With commercial MMPs

The fluorogenic substrate dye-quenched (DQ)-gelatin was purchased from Invitrogen (Carlsbad, CA, USA) and dissolved in water at 1 mg/mL. All solutions and dilutions were prepared in assay-buffer (50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% v/v Tween 20). A 96-well micro-assay plate (chimney, 96-well, black) was used. Each well was loaded with 0.1 mM (for a final volume of 200 µL) MMP-9 (Sigma), to which 100 µg/mL protein of total *Aloe* extract (for a final volume of 200 µL) was added, and the plate was incubated for 1 h at 37 °C. Subsequently, DQ-gelatin (at a final concentration of 2.5 µg/mL) was added to each well and the plate was allowed to incubate again, for 1 h. Fluorescence levels were measured read in a spectrophotometer Syenery HT, Bio-TEK (ex. 485 nm/em. 530 nm). In each experiment, both positive (no protein fraction) and negative (no enzyme) controls were included for all samples, to correct for possible proteolytic activities present in the *Aloe* extracts. All data were corrected by subtraction of their corresponding negative controls.

With HT29 cell culture extracellular media

The same method described above was used, with some alterations. Roughly, each well was loaded with 100 µL of extracellular HT-29 media (containing MMP-9 and MMP-2) after exposure to the *Aloe* extracts. Subsequently, DQ-gelatin (at a final concentration of 2.5 µg/mL) was added to each well (for a final volume of 200 µL) and the plate was allowed to incubate again, for 1 h. Fluorescence levels were measured read in a spectrophotometer Syenery HT, Bio-TEK (ex. 485 nm/em. 530 nm).

2.5.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Aloe samples were treated with 100 mM Tris-HCl buffer, pH 6.8, containing 100 mM β-mercaptoethanol, 2% (w/v) SDS, 15% (v/v) glycerol and 0.006% (w/v) *m*-cresol purple, and heated at 100 °C for 5 min. One-dimension electrophoresis was carried out, following the method described by Laemmly *et al.* (1970) [92] in a 12,5% (w/v) acrylamide resolving gel and a 5% (w/v) acrylamide stacking gel, and performed in a vertical electrophoresis unit at 100 V and 20 mA per gel. Gels were fixed for 20 min in 10% (w/v) TCA, and stained in 0.25% (w/v) Coomassie Brilliant Blue R-250, 25% (v/v) 2-propanol and 10% (v/v) acetic acid. Destaining was carried in a solution of 25% (v/v) 2-propanol and 10% (v/v) acetic acid.

2.5.3 Reverse gelatin zymography

Reverse zymography, used to detect and quantify MMPI proteins in different samples was performed as described in Hawkes *et al*. (2001) [93], with some modifications. Protein samples were treated with zymographic buffer (313 mM Tris-HCl buffer, pH 6.8, containing 10% (w/v) SDS, 50% (v/v) glycerol and 0.05% (w/v) bromophenol blue) and loaded in SDS-polyacrylamide (12.5% w/v acrylamide) gels copolymerized with gelatin (1% w/v) and 1 µmol/mL MMP-9. Electrophoresis was performed as described in 2.5.3 and the gels were washed three times in 2.5% v/v Triton X-100, for 60 min each, to remove SDS. Gels were then incubated overnight at 37 °C, with developing buffer (50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 0.01% w/v sodium azide),

stained with Coomassie Brilliant Blue G-250 0.5% (w/v) in 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, and destained with a solution of 50% (v/v) methanol, 10% (v/v) acetic acid in water. Dark bands visible against a white background marked the MMPI-mediated inhibition of gelatin degradation [93].

2.6 Statistical analysis

All experiments were performed in triplicate, in at least three independent times and the data are expressed as the mean ± standard deviation (SD). SigmaPlot software (version 12.5) was used for comparing different treatments, using one-way and two-way analysis of variance (ANOVA). Tukey's test was used to compare differences between groups and the statistical differences with P value less than 0.05 where considered statistically significant.

3 - Results and Discussion

3.1 Selected species: *A. vera* and *A. arborescens*

Evidence has shown that certain bioactive phytochemicals are often present in more than one *Aloe* species [82], an observation which inevitably supports the species-substitution hypothesis in traditional medicine. Among the hundreds of identified *Aloe* species, there are two which stand out as the most common in European countries, specifically in Portugal. *A. vera*, because of its huge popularity, and *A. arborescens*, because it is well adapted to the Portuguese climate and is widely spread across the public gardens as an ornamental plant. Fig. 3.1 shows representative images of these two species, present in the herbarium collection at the Instituto Superior de Agronomia.

Figure 3.1 - Representative photos of the selected species *A.arborescens* and *A. vera*, from the herbarium collection at the Instituto Superior de Agronomia and during the flowering season.

Although there are many differences between both species, such as morphological features, flower color, sap color, smell and other histological features, both species are often mistaken, as many media and anecdotal reports show. Although there are several articles highlighting the pytochemical composition of *Aloe vera*, much less is known about other species, like *A. arborescens*. Nonetheless *A. arborescens* has been the target of some studies in the last years, some of which related to its anti-cancer properties [16-18]. For example, *A. arborescens* was already proven to be effective in aiding chemotherapy when given orally at a dose of 10 mL thrice daily of a mixture consisting of 300 g of fresh leaves in 500 g of honey plus 40 mL of 40% (v/v) alcohol, every day without interruption, either during or after chemotherapy [16]. In this work we aimed to compare the potential anticancer activities between these two well-known *Aloe* species, *A. vera* and *A. arborescens*. With that in mind, we set out to quantify the compounds with higher potential in cancer inhibition.

3.2 Bioactive compounds in *A. vera* and *A. arborescens*

The literature describes several compounds in *A. vera* [82] which can be responsible for the various pharmacological activities previously described. The main phytochemical groups present in *A. vera* have been described as phenolic compounds (anthraquinones, anthrones), carbohydrates and proteins [7, 21, 79, 82-84]. The main components related to bioactivity in cancer and are summarized in Table 3.1.

Table 3.1 – Summary of the potential bioactive compounds present in *Aloe vera* leaves (Adapted from Park *et al*. (1998) and Hamman *et al*. (2008)).

Class	Components
Phenolic Compounds	*Aloe*sin, 8-*C*-glucosyl-7-*O*-methyl-(S)-*Aloe*sol, neo*Aloe*sin, 8-*O*-methyl-7-hydroxylaloin A and B, iso*Aloe*resin D, aloin A and B, *Aloe*resin E, *Aloe*-emodin (all identified in *A. vera*); *Aloe*nin, *Aloe*nin B and 10-hydroxylaloin A (specifically identified in *A. arborescens* by Park *et al*. (1998) [21]) **Effects in cancer:** antiproliferative and cytotoxic effects [16]
Carbohydrates	Pure mannan, acetylated mannan, acetylated glucomannan, glucogalactomannan, galactan, galactogalacturan, arabinogalactan, galactoglucoarabinomannan, pectic substance, xylan, cellulose [7] Mannose, glucose, **L**-rhamnose, aldopentose [7] **Effects in cancer:** immunomodulatory activity and stimulate necrosis and regression of tumors [25, 26]
Proteins	Lectins, lectin-like substance [7] **Effects in cancer:** prophylactic effect, regressed tumor size [94]

During evolution, these plants naturally evolved to produce important secondary metabolites in order to survive their environment and its predators [95]. These different metabolites or phytochemicals may act individually, additively or in synergy to improve the plant's health and its chances of survival in a given environment. Furthermore, it has been suggested that these combined actions of the phytochemicals usually tend to increase the bioactivity of the main medicinal constituents by influencing its assimilation in the body [77, 95]. Because of that, in this work, we not only aimed to compare the amounts of the potentially bioactive compounds in the two selected species, but we also wanted to select the best extraction procedure, that would allow the highest yield in these compounds. Therefore, we set out to quantify the major classes of bioactive compounds: proteins, total phenolic compounds and specifically anthraquinones, and total polysaccharides.

3.2.1 The amount of bioactive compounds extracted from *A. arborescens* and *A. vera* is influenced by the species and by the extraction procedure

The amounts of the different classes of bioactive compounds in both *Aloe* species are present in Fig. 3.2. When comparing *A. vera* and A. *arborescens* we can easily identify differences between species regarding both the extraction methods for each component and the amount of each component per amount of fresh weight.

Proteins

Aloe plants contain around 95% (w/v) water but less than 0.1% (w/v) protein [94]. Although the amount of total proteins present in *Aloe* is relatively small, its biological activities are meaningful as evidenced by their many applications, their major part related to wound healing and several skin diseases treatment [96-99]. Lectins are a class of proteins which have been recently identified in *Aloe* and have been pointed out as important anti-cancer agents, particularly in prevention [94].

The different extractions yielded different types proteins, according to their solubility: water-soluble proteins, which are extracted in 100 mM Tris-HCl buffer, and the water insoluble proteins, which encompass the membrane and wall-bound proteome and are extracted with methanol. It is important to note that in the protein quantifications, all samples were previously treated with PVPP to remove the majority of the phenolic components from the extract, which would otherwise interfere with protein quantifications. For the same reason, protein quantifications were also determined according to the method described by Bradford [85] because of the same reason. Results presented in Fig. 3.1a corroborate the low amount of proteins present in both species, which were overall less than 0.1% (w/v) of fresh weight. Nonetheless, protein amounts and distribution varied significantly among both species.

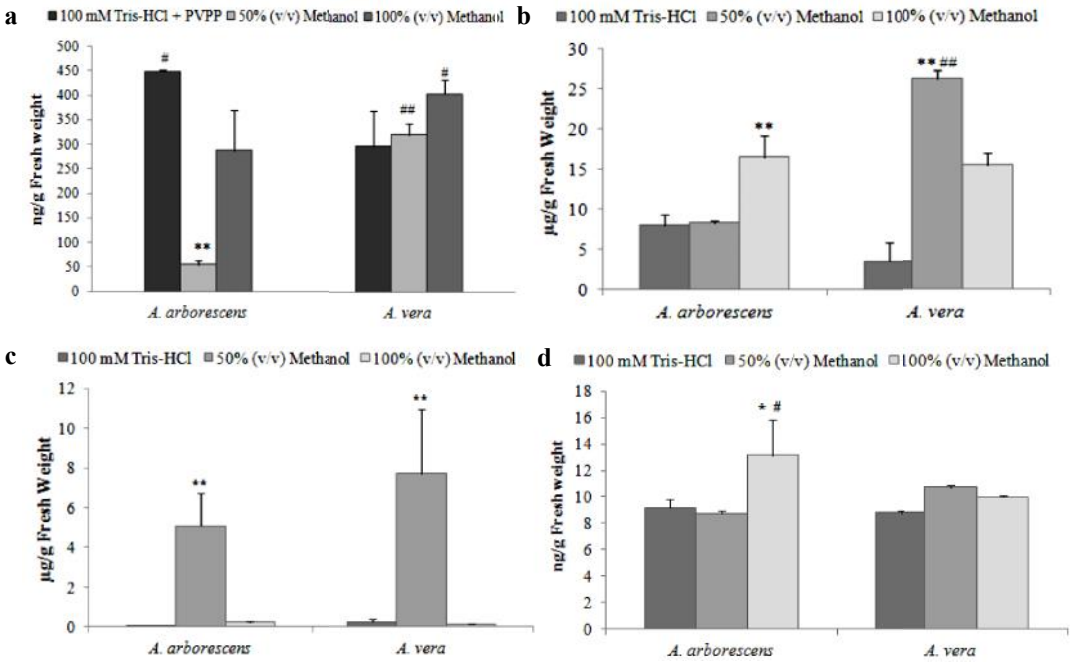

Figure 3.2 – Quantitative characterization of *A. vera* and *A. arborescens* considering the main phytochemical groups defined by Hamman *et al*. 2008 [7]. Each extraction was performed with 20g of fresh *Aloe* leaves for each of the three solvents: 100 mM Tris-HCl buffer, 50% (v/v) methanol and 100% (v/v) methanol. (a) Protein quantification using the Bradford method described by Bradford *et al*. (1976). All samples were treated with PVPP to eliminate the phenolic compounds that could interfere with the extraction of soluble proteins. (b) Phenolic compounds quantification with the Folin-Ciocalteau reagent and using gallic acid as standard. (c) Total carbohydrates quantification using phenol/sulfuric acid method and mannose as the standard. (d) Specific anthraquinone quantification (phenol component) using benzene, ammonia solution and *Aloe blue curacao* aloin.* P<0.05; **P<0.001 when compared between extracts of the same species; # P<0.05; ## P<0.001 the same extract when compared between species.

Whilst the amount of soluble protein was significantly higher (P<0.05) in *A. arborescens* (430 ng/g) than in *A. vera* (380 ng/g), *A. vera* presented the higher amounts of insoluble proteins than *A. arborescens* (404.61 ng/g and 288.3 ng/g, respectively) (P<0.05).

Phenolic compounds

Phenolic compounds were already described as the second major class of compounds found in *A. vera* [100], second only to polysaccharides (Fig. 1.3.b). Phenolic compounds present in plants, result from their secondary metabolism and are the main group of substances responsible for most of the plant medicinal properties, including *Aloe* species [27, 100, 101]. Regarding the results in Graphic 3.1.b, corresponding to total phenols present per extraction, it is clear that the extraction with 50% (v/v) methanol yields higher amounts of total phenolic in *A. vera* extraction (26.19 µg/g), whereas for *A.*

arborescens the extraction with 100% (v/v) methanol yielded the highest amounts (16.48 µg/g), in a significant manner ($P<0.05$). Albeit in Fig. 1.3.b *A. vera* presented more phenolic compounds ($P<0.05$) when compared to *A. arborescens*, when evaluating the specific group of anthraquinones (Fig. 1.3.d) *A. arborescens* had a significant higher content in the methanolic extractions.

Total carbohydrates

It is well-known that polysaccharides are abundantly represented in *A.vera*, being the major compound class present in the mucilage of these plants [25, 26]. Partially acetylated mannan or acemannan have been identified as the primary polysaccharides which are associated to the beneficial effects of *A. vera* as an anticarcinogenic agent. Acemannan for example was found responsible for the stimulation of the immune response in cancer scenarios, contributing to tumor weight reduction and the improvement of chemotherapy drugs [102, 103].

In the present work, regarding the composition in total polysaccharides between the two species in this work, the extraction with 50% (v/v) methanol presented a significantly higher yield than the other two extractions ($P<0.001$). For example, *A.vera* contains 7.72 ng/g against 0.25 ng/g for 100 mM Tris-HCl buffer and 0.14 ng/g for 100% (v/v) methanol. Similar results were found between both species for all tree extractions ($P>0.05$).

Overall, these results suggest that the composition in bioactive compounds is similar in both species, particularly in the phenolic compounds and total polysaccharides; there are significant differences that could be important for their bioactivities, namely the higher amount of anthraquinones and water-soluble proteins in *A. arborescens* when compared to *A. vera* extracts. *A. vera* nonetheless presented higher amounts of phenolics and wall and membrane-bound proteins.

3.2.2 The majority of the phytochemical components from *A. vera* are better extracted in 50% (v/v) methanol

When considering the whole leaf as an organ for phytochemical extraction, the possibility to combine several components has been suggested to potentiate its therapeutic activity [77, 95]. However, the choice of the extraction method by which the compounds are obtained is essential. Results here presented show that the extraction method not only influences the amount of bioactive component extracted but it also differs among species. Hence, for *A. vera*, for all compounds classes except the proteins, the majority of the compounds were better extracted with 50% (v/v) methanol (phenolics and polysaccharides, with $P<0.05$) whereas for *A. arborescens* the highest yields were obtained in the 100% (v/v) methanol extraction. This suggests the presence of different compounds but more importantly, it arises the question of whether most literature using non-aqueous polar and non-polar solvents such as ethanol, acetone etc. are using the correct extractions to yield the better bioactivities with *A. vera*.

3.3 Effects of total *Aloe* extracts in colon cancer cells

An estimated 3.45 million new cases of cancer and 1.75 million deaths from cancer occurred in Europe in 2012 [104], with colorectal cancer (CRC) being the second most common cause of cancer death in the European Union [104, 105]. CRC is often highly metastatic and resistant to anticancer treatment strategies [106], and despite the intensive research made and the significant advances in diagnosis, screening and treatment, the overall long-term outcome in patients has not significantly changed in the last decades; the five-year survival rate is approximately 60% [49 106]. MMP-9 inhibitors

(MMPIs) are considered anti-angiogenic agents and metastasis deterrents for CRC, and have also been demonstrated to effectively inhibit pre-cancer states such as colitis and other inflammatory bowel diseases [107]. In this work, we aimed to test and compare the anticancer and MMP-9 inhibitory potential of the selected extracts of both *Aloe* species under study. Hence, we selected the colon cancer cell line HT29 for our tests, using the standard cell proliferation and cell migration assays.

Since the all extraction procedures seemed to provide different amounts of different compounds (Fig. 3.1), and differed between species, preliminary experiments were conducted to select the best extraction procedure to use in the HT29 cell assays. Preliminary results (see annex 1) showed that using the cell migration wound assay, for *A.vera* the best results were obtained for 50% (v/v) methanol extractions, whereas for A. *arborescens* there were no significant differences between 50% (v/v) or 100% (v/v) methanol extracts. Therefore, the 50% (v/v) extraction was selected to pursuit our studies.

3.3.1 *Aloe* extracts reduce cancer cell growth

To test if the effect of the *Aloe* extracts was cytotoxic to HT29 cells, and if it influenced cell growth, we studied identical concentrations of plant extracts using a standard cell proliferation assay. Figure 3.2 illustrates the proportion of HT29 living cells after growth in the presence of different concentrations of *Aloe* extracts (50, 25 and 10 µg dry weight/mL), determined after staining with MTT (which can only be metabolized by living cells). The results show that a 2-day exposure to the 50% (v/v) methanol extract from both *Aloe* species did induce a significant reduction ($P>0.001$) in cancer cell growth when compared to controls.

Furthermore, this reduction was strongly dose-dependent, with higher concentrations inducing a significantly higher inhibition than the previous ($P<0.05$). Previous reports have shown that *Aloe* induces a direct inhibition of cancer cell proliferation through *Aloe*nin-like molecules. According to Lissoni *et al*. (2009) [16], this finding is not surprising, since *Aloe*nin and other similar molecules may be classified within the group of anthracenic and anthraquinonic substances, whose antiproliferative cytotoxic effects are well known [16].

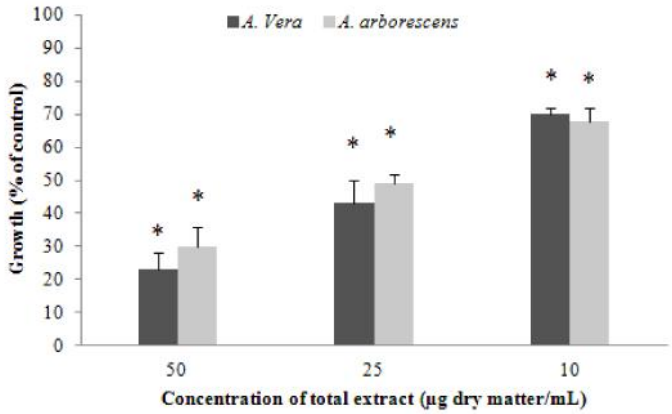

Figure 3.3 - Cell proliferation assay. Cells were exposed for 48h to *A. vera* and *A. arborescens* extracts prepared with 50% (v/v) methanol at for 48h. Cell growth was determined by the standard MTT assay. Results are expressed as the means of at least three replicate experiments ±SD.*$P<0.05$ between concentrations.

Interestingly, although it has been suggested that *A. vera* is the *Aloe* species with the lowest toxicity, our results show that there were no significant differences ($P<0.001$) between *A. vera* and *A. arborescens* in all concentrations tested. Being dose-dependent, the results point to a EC50 of 25 µg dry weight/mL for the 50% (v/v) methanol extracts. Minimal inhibitory concentrations were also determined and results are expressed in Table 3.2.

Table 3.2 - Minimal inhibitory concentrations for cell growth when HT29 cells were exposed to different concentrations of *A. vera* and *A. arborescens* extracts prepared with 50% (v/v) methanol. Results are expressed in µg dry weight/mL

	MICs (µg/mL)
A. vera	6,25
A. arborescens	3,12

MIC results point to much lower inhibitory concentrations, which were nonetheless higher inhibitory activity for *A. arborescens*, with a MIC of 3.12 µg dry weight/mL, which is roughly half the MIC determined for *A.vera*,

3.3.2 Aloe extracts differently reduce cancer cell invasion

Whilst the cell proliferation assay provides us with growth inhibition, cell metabolism and overall cytotoxicity, the wound healing assay helps to evaluate the reaction of confluent cells in response to a disruption of cell-cell contacts. What normally occurs is an increase of the growth factors concentration at the wound margins which stimulates proliferation and migration in order to close the opened wound [108], thus mimicking the metastatic process of cell invasion. These two processes are extremely important when considering cancer development and have been closely related to MMP activity, particularly MMP-9.

In the wound healing assay we set out to characterize the migratory response of HT-29 cells when exposed to the 50% (v/v) methanol extractions of *A.vera* and *A. arborescens*. Since the EC50 concentration was 25 µg/mL, we selected this concentration for the wound healing assays and the results are expressed in Fig. 3.3. The percentage of wound closure of cells exposed to the *Aloe* extracts was compared to a positive control which presented a maximum closure of 74% after 48h and to a negative control of a known MMP-9 inhibitor, doxycycline at a concentration of 40 µg/mL. This concentration was selected based on previous reports which show doxycycline inhibiting MMP-9 activity in other cancer scenarios [107].

Results show that all treatments induce very significant differences ($P<0.001$) when compared to controls. Unlike the cell proliferation assays, significant differences were observed between the *A.vera* and *A.arborescens* extracts ($P<0.05$). While *A. arborescens* presented a wound closure of 19% , which was not significantly different ($P>0.05$) from the deoxycycline treatments, *A. vera* was less effective in inhibiting cell invasiveness with 37% of wound closure, which was significantly higher than deoxycycline [109]. These results suggest that although *A.vera* is the most used species and has been attributed a lot of therapeutic benefits, regarding anti-cancer potential, it may not be as efficient as it has been described in reducing cancer cell invasion. On the other hand, *A.arborescens* has also been proven to have very good results when used as a co-adjuvant in some chemotherapeutic treatments [16] and this might be due to its capacity to inhibit tumor invasion and cell migration [109, 110].

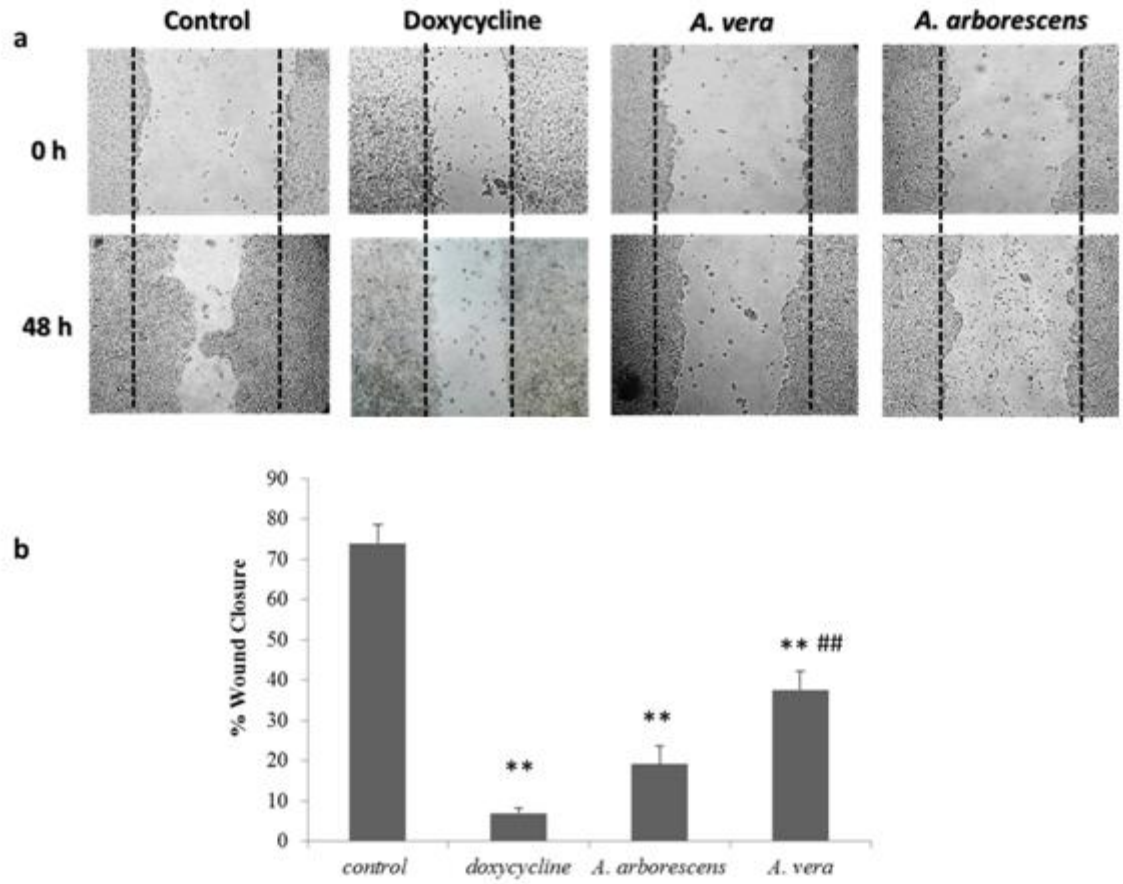

Figure 3.4 – (a) Representative images of the wound healing assay on cells exposed to *A. vera* or *A. arborescens* extractions with 50% (v/v) methanol at time 0h and after 48h of exposure. (b) Wound closure after a 48h period exposure to *A.vera* or *A.arborescens* extractions with 50% (v/v) methanol. Doxycycline at a concentration of 40 μg/mL was used as a positive control for metalloproteinase inhibition (Kim *et al*. 2005). * P<0.05; **P<0.001 when compared with positive controls; # P<0.05; ## P<0.001 when compared to doxycycline.

3.4 *Aloe* extracts inhibit gelatinase activity

MMPs have been implicated in the migratory ability of cancer cells due to its capacity to degrade the extracellular membrane. MMP-2 was the first endopeptidase recognized to degrade collagens and to be associated with the invasive and metastatic potential of cancer cells [108]. The active form of MMP-2 co-localize with a pro-form of MMP-9 in many types of cancer, being able to activate it, thus increasing tumor malignancy [111, 112]. MMP-9 is associated to promotion of metastization, angiogenesis and cell survival [112, 113, 114].

Since *A. vera* has been pointed to concomitantly present anti-inflammatory and anti-cancer activities, it seems plausible to infer that MMP-9 (a known key player in both conditions) can also be a target for *Aloe*'s bioactive compounds. Nonetheless, albeit there are many works on the anticancer activities of *A. vera*, to our knowledge, there are no studies which correlate its anticancer activities with MMP-9 and MMP-2 inhibition. Also, although there has been a considerable body of research related to the effects of *A. vera* on cancer cells, very few use the whole extract, and even fewer have tested *A. arborescens*. Under this context, in this work, it was also our goal to test the activities of MMP-9 and MMP-2 in the extracellular media of the HT29 cells after exposure to 25 μg/mL, using the DQ-gelatin kit. This kit contains a quenched-die gelatin substrate, which when under proteolytic attack, for example, in the presence of active forms of either one of the gelatinases, produces

fluorescence. Fig. 3.5 shows the total gelatinolytic activity present in the extracellular media expressed as a percentage of controls.

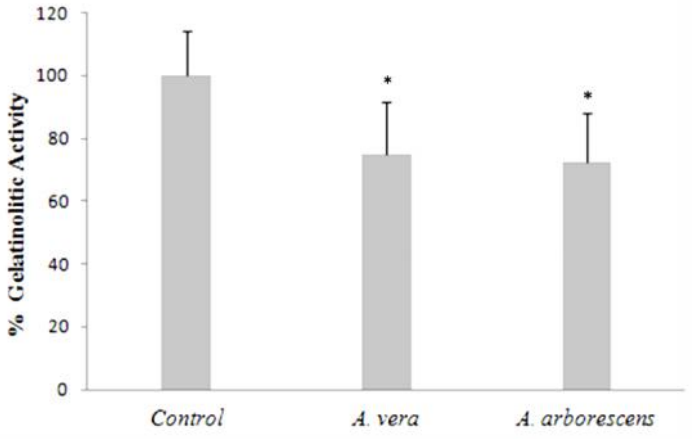

Figure 3.5 - MMP-9 and MMP-2 activities are inhibited by *A. vera* and *A. arborescens* extracts. Total gelatinolytic activity in cell media was evaluated after the 48 h incubation of HT29 cells with *Aloe* extracts, using the fluorogenic gelatin assay, DQ™-gelatin (Life Technologies), where an increase in fluorescence is proportional to enhanced gelatin degradation, caused by MMP-9 and MMP-2 activities. Values are expressed as percentages relative to controls and are represented as the media ± SD of at least three replicate experiments. * $P<0.05$.

Figure 3.5 suggests that both *Aloe* species could indeed inhibit the gelatinase activity in the cell growth media, but only up to 20% being significantly similar between both species. Since both gelatinases MMP-9 and MP-2 are present simultaneously in the extracellular media and since there could be specificity towards one of them, we further used gelatin zymography. This technique uses a regular electrophoresis (SDS-PAGE) under non-reducing conditions, to separate the extracellular media proteins (containing MMP-9and MMP-2 among many others) by their mass, but contains imbibed in the acrylamide matrix gelatin, the substrate for gelatinases. After incubation, the active individualized gelatinases will induce proteolysis of the gelatin in the spot of the gel where they are located, producing a white band in contrast to an otherwise blue gel, whereas inactive (i.e. inhibited) gelatinases remain unaltered. Figure 3.6 shows examples of gelatin zymography of the samples used in this work.

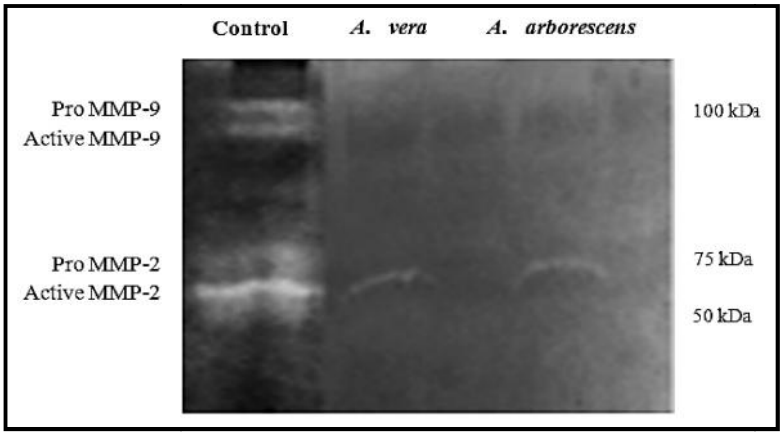

Figure 3.6 - MMP-9 and MMP-2 activities are inhibited by *A. vera* or *A. arborescens* extracts. Extracellular HT29 cell media samples were subjected to a non-reducing gel electrophoresis (SDS-PAGE) in 12,5 % polyacrylamide gels supplemented with 0.2% (w/v) gelatin from bovine skin, type B . After renaturation, MMP-9 and MMP-2 activity was stimulated by incubation in reaction buffer (see material and methods) and the gel was stained for total protein. The presence of gelatinase activity is observed by clear bands against a dark blue background.

White bands are indicative of the gelatinolytic activity of the specific bands, active MMP bands, including the pro-enzyme and its active form. In the case of the MMPs, they are usually synthesized as zymogens (pro-MMPs), with their catalytic activity blocked by a cysteine switch and are only activated by its removal, through limited proteolysis. In the zymography, the pro-gelatinases also become active because they are denaturated by the SDS, thus exposing the catalytic site (hence the slightly higher mass of the pro-enzymes in the zymography, which still maintain the short amino acid sequence of the cysteine switch). Hence there are two white bands for each MMP, as observed in the controls in Fig. 3.5 gelatinase A or MMP-2 has a 72kDa molecular mass dimension whereas gelatinase B or MMP-9 has 92kDa.

When observing the lanes of the extracellular media exposed to the *Aloe* extracts, we can see that, after exposure to the *Aloe* extracts, MMP-9 was completely inhibited in both forms, pro-enzyme and active form, whereas MMP-2 active form is still active, in both cases. These results suggest a stronger inhibitory specificity towards MMP-9, in both *Aloe* species. Since MMP-9 is strongly related to inflammation, wound healing and cancer invasion [41, 49, 50, 114], these results are more consistent to the reported bioactivities of *Aloe*. This may also explain why only 20% of the total gelatinase activity was inhibited. Indeed, since MMP-2 was still active in the extracellular extracts, it could still induce proteolysis of the quenched-die gelatin.

MMP-9 specificity can be of significant importance because has been assumed that most MMPIs are non-specific, and this is, in turn, responsible for their generalized adverse side-effects. A more specific inhibition, targeted solely to MMP-9, particularly in colon cancer, where this MMPI might act *in situ* may be of significant potential to anticancer and anti-inflammatory approaches in the gastrointestinal diseases.

In order to understand if the observed inhibitory activities were due to direct binding of *Aloe* extract components to MMP-9, we further tested its inhibitory activity towards these enzymes, using once again the DQ-gelatin kit. Fig. 3.7 shows the MMP-9 (Sigma) activities in the presence of both extracts, expressed in percentage of controls.

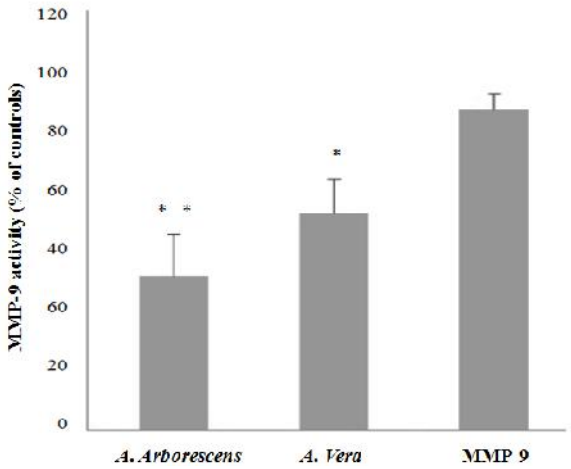

Figure 3.7 - Gelatinolitic activities of MMP-9 after exposure to ethanol extracts of several Aloe species. MMP-9 was allowed to incubate in the presence of 25 µg/mL extracts of *A. arborescens* and *A. vera* and its activity was quantified by the DQ-gelatin assay. Results are expressed as percentage of controls. Values are the means of at least 3 replicate experiments ± SD. *P<0.05, **P<0.001.

As observed in Fig. 3.7, both *Aloe* extracts were able to reduce MMP-9 activity directly, but this inhibition was higher for *A. arborescens* (P<0.001) than for *A.vera* (P<0.05). These results suggest that the higher MMP-9 inhibitory activities in *A. arborescens* can be the reason for the higher inhibition of cell invasion found for this species (Fig.3.3). However, the percentage of inhibition was not as high as the one observed in the zymographic analysis, where MMP-9 was completely inhibited.

This suggests a possible broader mechanism: the *Aloe* extracts do not only inhibit directly this enzyme, but also might alter its expression at the DNA level. Further work should be done in order to pursuit this hypothesis.

Nonetheless, overall, our general results suggest specific important issues: 1) the genus *Aloe* is able to inhibit MMPs, 2) there are indeed differences among *Aloe* species and 3) possibly, *A. vera* is not the most efficient *Aloe* species. These facts allow us to ask important questions: is all the debate and misconception about *A. vera* being caused by erroneous uses of different species (more or less efficient, or more toxic than *A. vera*)? And are we missing out on not testing other *Aloe* species, in search for important bioactivities, which can efficiently be used in medical treatments?

The fact that *Aloe* species reduce MMP activity is also an important factor MMP-9 and MMP-2, are proteolytic enzymes which can degrade the extracellular matrix components (ECMs) [64-68, 114] that is usually closely associated to inflammation, wound closure and also the formation of metastasis in several types of cancer [64-68]. Therefore, our discovery that *Aloe* species can reduce MMP activity is consistent to inhibition of specific problems such as wound healing, inflammation and cancer, which in fact match many of the proclaimed health benefits of *Aloe*, and scientific results obtained over the years. So the following question arises: is the activity of *Aloe* against MMPs the reason for its benefits? Can it be used to treat specific conditions? Further work should be pursued to answer these questions.

4 - Conclusions

Overall, the work presented in this thesis has shown that both studied species of *Aloe*, i.e. *A. vera* and *A. arborescens* present indeed a potential for inhibiting tumor growth and invasion, both by inhibiting cell growth and invasion. The results also show that this inhibition may be associated to a specific reduction of MMP-9 activity.

Despite having similar concentrations of various bioactive classes of compounds, *A. arborescens* appears to be a more effective cancer cell invasion inhibitor than *A. vera*. This might be due to a greater amount of bioactive compounds like anthraquinones present in *A. arborescens*, or to the presence of specific compounds not yet identified in this species. Nonetheless, results show that in studies related to cancer prevention or therapy using *Aloe* species, *A. arborescens* should be considered as an effective alternative to *A. vera*. Furthermore, our results also highlight the importance of the extraction procedure to obtain higher amounts of bioactive extracts, such as phenolics, anthraquinones and polysaccharides, particularly in *A.vera*.

5 - References

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Annex 1

1.1 Cell migration assay

For cell migration analysis, the wound healing assay was performed. HT29 cells ($5x10^5$ cells/well) were seeded in 6-well plates and allowed to reach to 80% confluence. Wounds were performed by making a scratch across the cell monolayer to create an open gap, mimicking a wound. Cells were then washed twice with PBS to remove floating debris. Each well was subsequently filled with fresh media containing the samples under study in a concentration of 100 μg/mL and allowed to grow for 48 h. The invaded area after 48 h was calculated in each treatment and compared to the initial area at 0 h, to determine the area covered by migrating cells into the denuded zone at the beginning of treatment. This comparison allowed us to assess the inhibitory effect exerted by each protein fraction on the HT29 cell migrating capacity.

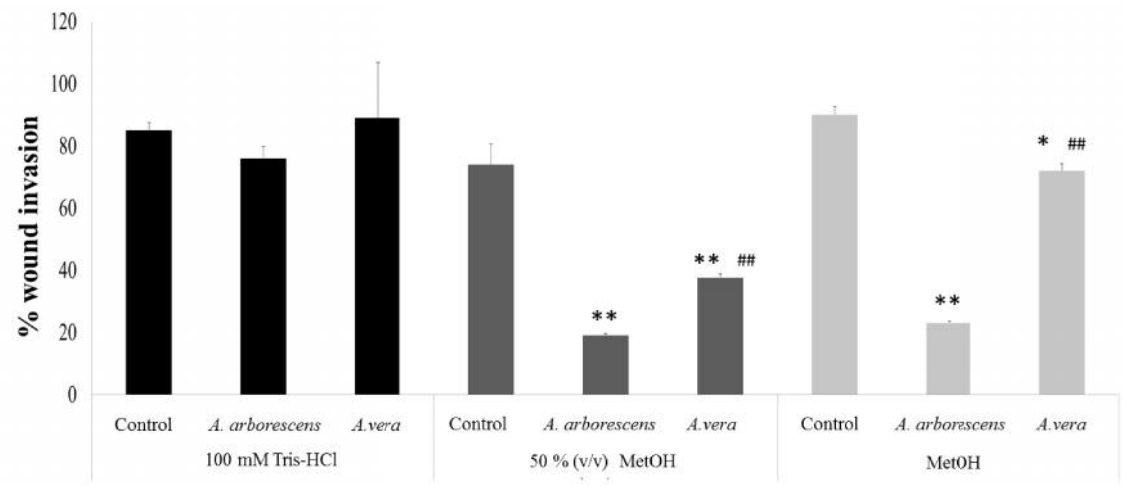

Figure 1.1 – (a) Representative images of the wound healing assay of cells exposed to *A. arborescens* or *A. vera* extracts prepared with 100 mM Tris-HCl, 50% (v/v) methanol and 100% (v/v) methanol at a time 0h and after 48h of exposure, (b) Wound closure after a 48h period of exposure to *A. arborescens* or *A. vera* extractions with 100 mM Tris-HCl buffer, 50% (v/v) methanol and 100% (v/v) methanol). * $P<0.05$; **$P<0.001$ when compared with controls on the same extraction; # $P<0.05$; ## $P<0.001$ when compared between the same species on the same type of extraction.

Results show that buffer-soluble extractions did not exert any significance reduction in cell migration when compared to controls ($P>0.05$), whereas in the other methanol extractions there was an overall reduction in cell invasion induced by both species. For A. *arborescens*, both methanol extractions induced a significant reduction ($P<0.001$) of around 80% in cancer cell inhibition, which was similar in both types of methanol extractions. In the case of *A. vera* there was a higher inhibition for 50% (v/v) methanol extracts when compared to the 100% methanol extract ($P<0.05$).

As there was no difference for *A. arborescens* and it provided the best results for *A. vera*, the 50% (v/v) methanol extraction was selected for the cancer cell assays.